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Multiplicity of 3-Ketosteroid-9α-Hydroxylase Enzymes in *Rhodococcus rhodochrous* DSM43269 for Specific Degradation of Different Classes of Steroids▽†

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The well-known large catabolic potential of rhodococci is greatly facilitated by an impressive gene multiplicity. This study reports on the multiplicity of *kshA*, encoding the oxygenase component of 3-ketosteroid 9α-hydroxylase, a key enzyme in steroid catabolism. Five *kshA* homologues (*kshA1* to *kshA5*) were previously identified in *Rhodococcus rhodochrous* DSM43269. These $KshA_{DSM43269}$ homologues are distributed over several phylogenetic groups. The involvement of these KshA homologues in the catabolism of different classes of steroids, i.e., sterols, pregnanes, androstenes, and bile acids, was investigated. Enzyme activity assays showed that all KSH enzymes with $KshA_{DSM43269}$ homologues are C-9 α-hydroxylases acting on a wide range of 3-ketosteroids, but not on 3-hydroxysteroids. KshA5 appeared to be the most versatile enzyme, with the broadest substrate range but without a clear substrate preference. In contrast, KshA1 was found to be dedicated to cholic acid catabolism. Transcriptional analysis and functional complementation studies revealed that *kshA5* supported growth on any of the different classes of steroids tested, consistent with its broad expression induction pattern. The presence of multiple *kshA* genes in the *R. rhodochrous* DSM43269 genome, each displaying unique steroid induction patterns and substrate ranges, appears to facilitate a dynamic and fine-tuned steroid catabolism, with C-9 α-hydroxylation occurring at different levels during microbial steroid degradation.

Rhodoccoci are capable of degrading a wide range of organic compounds (15, 32). This strong catabolic potential is encoded by an extremely large genome, of >9.7 Mb in the case of *Rhodococcus jostii* RHA1, which also carries numerous gene homologues for various enzyme classes (20). Multiple steroid catabolic gene clusters, for example, have been identified in *R. jostii* RHA1 (19, 20, 33). In particular, several homologous genes encoding key enzymes involved in steroid ring opening have been identified, i.e., 3-ketosteroid 9α-hydroxylase (KSH), encoded by *kshA* and *kshB*, and 3-ketosteroid Δ1-dehydrogenase (KSTD), encoded by *kstD* (13, 33). Hydroxylation of steroid substrates at the C-9 position, together with dehydrogenation of the A-ring performed by KSTD, leads to opening of the steroid polycyclic ring structure and the formation of 3-hydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione (3-HSA) (7, 31). Knowledge of steroid catabolic enzymes is limited, despite the fact that sterol-degrading rhodococci and mycobacteria are of great industrial and pharmaceutical interest (6, 9, 12, 17, 25, 32). In recent years, interest in steroid catabolic enzymes has gained momentum, following the discovery of cholesterol catabolic gene clusters in *R. jostii* RHA1 and in the human pathogen *Mycobacterium tuberculosis* H37Rv (33). Interestingly, *kshA* and *kshB* have been identified as essential factors in the pathogenesis of *M. tuberculosis* H37Rv (10).

KSH is a two-component enzyme system, consisting of a terminal oxygenase, KshA, and a ferredoxin reductase, KshB. The *kshA* and *kshB* genes, encoding KshA and KshB, respectively, were first identified in *Rhodococcus erythropolis* SQ1, a strain possessing at least 3 *kshA* homologues (31, 34). A crystal structure of KshA of *M. tuberculosis* H37Rv was recently elucidated and revealed that KshA of H37Rv likely functions as a trimer (α3) (4). KshB from *Rhodococcus rhodochrous* DSM43269 was shown to contain FAD as flavin cofactor and a plant-type Fe_2S_2 cluster (4, 24).

We previously reported the cloning, heterologous expression, and characterization of KshA and KshB from *R. rhodochrous* DSM43269 (IFO3338) (1) and showed that this KSH enzyme displayed activity toward a subtle range of saturated and unsaturated 3-ketosteroid substrates (24). We have also performed cloning of an additional four *kshA* homologous genes from *R. rhodochrous* DSM43269 and the construction of a 5-fold *kshA* null mutant strain, *R. rhodochrous* RG32, blocked in steroid B-ring opening (35a). To investigate whether each of these five homologous KshA proteins is capable of catalyzing steroid 9α-hydroxylation and to analyze their physiological roles in steroid catabolism, we performed functional complementation experiments with a *kshA* null mutant strain, RG32 of *R. rhodochrous* DSM43269, which is blocked in 4-androstene-3,17-dione (AD) degradation. Moreover, *kshA* transcriptional analyses of wild-type DSM43269 cells induced with a range of steroid substrates were performed. Finally, a biochemical characterization of heterolo-

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gously expressed and purified KSH enzymes was undertaken to determine the substrate range of the different KshA proteins involved.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* cloning strain DH5α (Stratagene) and expression strains *E. coli* BL21(DE3) (Invitrogen) and *E. coli* C41(DE3) (21) were grown in Luria-Bertani (LB) at 37°C, 200 rpm. Wild-type *R. rhodochrous* DSM43269 was obtained from the DSMZ culture collection. The construction of the 5-fold *kshA* mutant strain *R. rhodochrous* RG32 is described elsewhere (35a). *R. rhodochrous* strains were grown in LB broth or mineral medium at 30°C and 200 rpm. Mineral medium contained K_2HPO_4 (4.65 g liter^{-1}), $NaH_2PO_4 \cdot H_2O$ (1.5 g liter^{-1}), NH_4Cl (3 g liter^{-1}), $MgSO_4 \cdot 7H_2O$ (1 g liter^{-1}), and Vishniac stock solution (1 ml liter^{-1}). Vishniac stock solution was prepared as follows (modified from the methods described in reference 35): EDTA (10 g liter^{-1}) and $ZnSO_4 \cdot 7H_2O$ (4.4 g liter^{-1}) were dissolved in distilled water (pH 8.0, adjusted by using 2 M KOH). Then, $CaCl_2 \cdot 2H_2O$ (1.47 g liter^{-1}), $MnCl_2 \cdot 7H_2O$ (1 g liter^{-1}), $FeSO \cdot 7H_2O$ (1 g liter^{-1}), $(NH_4)_6\,Mo_7O_{24} \cdot 4H_2O$ (0.22 g liter^{-1}), $CuSO_4 \cdot 5H_2O$ (0.315 g liter^{-1}), and $CoCl_2 \cdot 6H_2O$ (0.32 g liter^{-1}) were added in that order at pH 6 and finally stored at pH 4.0. When appropriate, ampicillin, apramycin, or kanamycin was added to a final concentration of 100, 50, or 25 μg ml^{-1}, respectively.

Steroids. AD, 1,4-androstadiene-3,17-dione (ADD), 19-nor-4-androstene-3,17-dione (nordion), 3α-hydroxy-5α-pregnane-20-one, 5α-androstan-17β-ol-3-one (stanolon), 3β-hydroxy-5α-androstane-17-one, and 9α-hydroxy-4-androstene-3,17-dione were obtained from Schering-Plough (Oss, Netherlands). 17β-Hydroxy-4-androstene-3-one (testosterone), 11β-hydrocortisone, 3α-,7α-, 12α-trihydroxy-5β-cholan-24-ioc acid (cholic acid), 4-cholestene-3-one (cholestenone), and 5-cholestene-3β-ol (cholesterol) were obtained from Sigma-Aldrich, and 4-pregnene-3,20-dione (progesterone) was obtained from ICN Biomedicals Inc. 1-(5α)-Androstene-3,17-dione, 5α-androstane-3,17-dione, 5β-androstane-3,17-dione, 5α-androstane-17-one, and 23,24-bis-nor-cholesta-4-ene-22-oic acid (4-BNC) were obtained from Steraloids. 23,24-bis-nor-cholesta-1,4-diene-22-oic acid (1,4-BNC) was obtained at near-100% yield using 4-BNC as a substrate for the KSTD1 enzyme of *R. erythropolis* SQ1 (13, 30). The steroids were extracted from the assay mixture with ethyl acetate and dried by evaporation with N_2.

Phylogenetic tree construction. MEGA version 4.0.2 was used for the construction of a phylogenetic tree (14). The amino acid sequences used to construct the phylogenetic tree were obtained from GenBank databases (accession numbers are shown in parentheses) as follows: HMPREF0063_12465 (ZP_07718021) of *Aeromicrobium marinum* DSM 15272, BamIOP4010DRAFT_2656 (ZP_02890593) of *Burkholderia ambifaria* IOP40-10, BCAM1620 (YP_002234232) of *Burkholderia cenocepacia* J2315, Bcep18194_B1448 (YP_372206) of *Burkholderia* sp. 383, Gbro_0919 (YP_003272124) of *Gordonia bronchialis* DSM 43247, H16_B0672 (YP_728834) of *Ralstonia eutropha* H16, MAB_0080c (YP_001700834), MAB_3627c (YP_001704355), and MAB_4173 (YP_001704900) of *Mycobacterium abscessus* ATCC 19977, MAV_0633 (YP_879913) and MAV_3037 (YP_882223) of *Mycobacterium avium* 104, Mb3556 (NP_857195) of *Mycobacterium bovis* AF2122/97, Mflv_1533 (YP_001132803), Mflv_1546 (YP_001132816), and Mflv_4566 (YP_001135822) of *Mycobacterium gilvum* PYR-GCK, MintA_010100002014 (ZP_05223666) and MintA_010100021954 (ZP_05227611) of *Mycobacterium intracellulare* ATCC 13950, Mjls_0287 (YP_001068591) of *Mycobacterium* sp. JLS, MkanA1_010100023803 (ZP_04751020) of *Mycobacterium kansasii* ATCC 12478, MMAR_5015 (YP_001853274) of *Mycobacterium marinum* M, Mmcs_0296 (YP_637473) and Mmcs_4640 (YP_641800) of *Mycobacterium* sp. MCS, MSMEG_2870 (YP_887190) and MSMEG_5925 (YP_890151) of *Mycobacterium smegmatis* strain MC2 155, KshA RV3526 (NP_218043) of *Mycobacterium tuberculosis* H37Rv, MUL_4089 (YP_907606) of *Mycobacterium ulcerans* Agy99, Mvan_2869 (YP_953681), Mvan_5211 (YP_955988), and Mvan_5225 (YP_956002) of *Mycobacterium vanbaalenii* PYR-1, Nfa33560 (YP_119567), Nfa4750 (YP_116681), Nfa22480 (YP_118459), and Nfa33740 (YP_119585) of *Nocardia farcinica* IFM 10152, Noca_2518 (YP_923709) of *Nocardioides* sp. JS614, RALTA_B0514 (YP_001796945) of *Cupriavidus taiwanensis*, RER_07540 (YP_002764201), RER_09150 (YP_002764362), RER_13800 (YP_002764827), and RER_51130 (YP_002768560) of *Rhodococcus erythropolis* PR4, Reut_A1576 (YP_295786) of *Ralstonia eutropha* JMP134, REQ_06790 (YP_004005481), REQ_08980 (YP_004005694), REQ_15470 (YP_004006310), REQ_40110 (YP_004008669), REQ_42740 (YP_004008918), REQ_43730 (YP_004009014), and REQ_45190 (YP_004009156) of *R. equi* 103S, RHOER0001-0749 (ZP_04388293), RHOER0001_0834 (ZP_04388135), RHOER0001_2900 (ZP_04382763), and RHOER0001_4808 (ZP_04386887) of *Rhodococcus erythropolis* SK121, KshA1 (AAL96829) and KshA2 (ACD11366) of *Rhodococcus erythropolis* SQ1, KshA Ro04538 (YP_704482), KshA2 (*Ro02490*; YP_702453), KshA3 (*ro05811*; YP_705746), and KshA4 (*ro09003*; YP_708205) of *Rhodococcus jostii* RHA1, ROP_22170 (YP_002779409), ROP_44530 (YP_002781645), and ROP_58730 (YP_002783065) of *Rhodococcus opacus* B4, KshA1 (ADY18310), KshA2 (ADY18316), KshA3 (ADY18318), KshA4 (ADY18323), and KshA5 (ADY18328) of *Rhodococcus rhodochrous* DSM43269, Sare_2880 (YP_001537697) of *Salinispora arenicola* CNS-205, SSMG_02043 (ZP_07278003), and SSMG_04623 (ZP_07280583) of *Streptomyces* sp. AA4, Strop_2672 (YP_001159494) of *Salinispora tropica* CNB-440, Tcur_3509 (YP_003301083) of *Thermomonospora curvata* DSM 43183, and Tpau_3849 (YP_003648763) of *Tsukamurella paurometabola* DSM 20162.

Electrotransformation of *R. rhodochrous* strain RG32 with pRRE1-derived plasmids for functional complementation with *kshA* genes. Competent cells of strain RG32 were prepared by growing cell cultures in 250 ml LB broth to an optical density at 600 nm (OD_{600}) of 0.2 to 1.0. The cultures were incubated on ice for 1.5 h. All further steps were done at 4°C unless stated otherwise. Cells were pelleted by centrifugation for 10 min at 5,000 × *g*. The cells were washed twice with MilliQ (125 ml), centrifuged (10 min, 5,000 × *g*), washed in 10% glycerol (25 ml), and centrifuged for 10 min at 2,000 × *g*. Cells were resuspended in 10% glycerol (1 ml) and stored in 100-μl aliquots at −80°C until use. For electrotransformation, cells were thawed on ice, and 1 μl (0.1 to 0.4 μg) of plasmid DNA (pA1rho9, pA2rho5, pA3rho5, or pA4rho15 [see Table S1 in the supplemental material]) was added to 100 μl of competent RG32 cells, mixed, and incubated for 1 min on ice. The cells were pulsed at 2.5 kV, 25 μF, and 1,000 Ω (field strength, 12.5 kV cm^{-1}). LB broth (1 ml) was added, and the cells were incubated for 4.5 h at 30°C and 200 rpm prior to plating on selective LB agar supplemented with apramycin.

Growth on different steroids as a sole carbon and energy source. LB-grown precultures of *R. rhodochrous* strains were used to inoculate (1:100) 25 ml of mineral liquid medium supplemented with representatives of different classes of steroids, i.e., androstenes, sterols, pregnanes, and bile acids, using 0.5 g liter^{-1} steroid (AD, cholesterol, progesterone, and cholic acid). Cholesterol was added as a solid to the medium, autoclaved, and dispersed by sonication. The other steroids were dissolved in dimethyl sulfoxide as 25-mg ml^{-1} stock solutions and then added to the medium. Growth was followed for several days based on OD_{600} measurements of cell cultures supplemented with AD or cholic acid. Due to the low solubilities of progesterone and cholesterol, total protein content was used to quantify turbidity in cell cultures supplemented with these steroids. Cell cultures (500 μl) were pelleted by centrifugation and resuspended in 100 μl bacterial protein extraction reagent (B-PER; Thermo Scientific). After 5 min, 400 μl MilliQ was added and the mixture was vigorously vortexed and incubated for 10 min. An aliquot of 160 μl was mixed with 640 μl MilliQ and 200 μl protein assay reagent (Bio-Rad). Bovine serum albumin (BSA) was used as a standard to determine the protein content of the sample.

Cholesterol bioconversions. Cultures of *R. rhodochrous* strains (25 ml) were grown to an OD_{600} of approximately 4 in LB broth. Cholesterol (1 g liter^{-1}) was added, and samples (0.5 ml/culture) were taken at different time points for analysis of formed products by high-performance liquid chromatography (HPLC) as described below.

Transcriptomic analysis of *R. rhodochrous kshA* homologues in steroid-induced cell cultures. Cultures of *R. rhodochrous* DSM 43269 were grown in mineral medium (100 ml) supplemented with sodium acetate (2 g liter^{-1}) to an OD_{600} of approximately 2. The cultures were then induced with 1 g liter^{-1} steroid (AD, cholesterol, progesterone, or cholic acid). Noninduced cultures were used as negative controls. The cultures were induced for 4 h in the cases of AD, progesterone, and cholic acid and for 24 h in the case of cholesterol. After centrifugation for 30 min at 10,000 × *g*, pelleted cells were frozen in liquid nitrogen and crushed in an ethanol (70%)-cleaned mortar. Crushed cells were stored at −80°C. RNA was isolated from the crushed cells using the RNeasy kit (Qiagen). An on-column DNase treatment was performed by adding DNase solution to a column that consisted of 40 μl yellow core buffer (Promega), 5 μl 0.09 mM $MnCl_2$, and 5 μl recombinant DNase I (Roche). After incubation at room temperature for 15 min, 200 μl of stop solution (Promega) was added. RNA was eluted in 40 μl MilliQ, subsequently treated with Turbo DNase (Ambion), and stored at −80°C. PCR was performed on RNA to verify that all DNA had been removed. If necessary, DNase treatment was repeated until all DNA was removed from the RNA samples. The PCR mixture (25 μl) consisted of 50 ng RNA, 1× polymerase buffer, 4.0 mM $MgCl_2$, deoxynucleoside triphosphates (0.2 mM), primers (0.2 μM; Table 1), *Taq* polymerase (1 U; Roche), and MilliQ under the following conditions: 5 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 68°C, and 1 min at 72°C; and 7 min at 72°C. Reverse transcription-PCR (RT-

TABLE 1. Primers used in this study

Target	Primer name	Product size (bp)	Sequence (5′–3′)
kshA1	KshARho-1	1,191	F, CATATGAGCCTCGGCACTTCCGAA R, GCGCTAGCCCGCGGTGGTGGACT
kshA2	KshaRho-2	1,214	F2, CATATGGCACCGTGGGTTCCAC R, GCGTCAGGATCCCGATTCGGCGG
kshA3	KshaRho-3	1,164	F, GCGCATATGGACATGGCACAGATT R, TCGTCACACCTCCGCTTCCTGCTT
kshA5	KshaRho-5	1,179	F, CATATGTCCATCGACACCGCACG R, GCTCTAGGGGGTCGCGGTGGAGC
kshA1	KshA1Rho-RT	374	F, AACGGCCGCTGCAAGAACATC R1, TTGACCGTTCGCGTGCGGAC
kshA2	KshA2Rho-RT	338	F2, CCGTTCCGGAGGTTCCCACC R2, TCGGCGTCGACTCGGGATCA
kshA3	KshA3Rho-RT	371	F, AACGGCAAGTGCACGGACATC R1, CTGCGTCGCCATGCCCTTGT
kshA4	KshA4Rho-RT	333	F1, CGAAGCTCGGCCGTACCAAG R1, ATCTGCACACCGGTCTTGAT
kshA5	KshA5Rho-RT	407	F, GCCTGGACGACCCTCGAACGC R1, GTCTGGCCGTTGGCATCGGA
16S RNA	16SF167Ameth 16SR483Ameth	350	GTGGCCTACCAAGGCGACGA AGACGCGACAAGCCGCCTAC

PCR) was performed using a SuperScript III Platinum one-step qRT-PCR kit (Invitrogen). The mixture (25 μl) contained RNA (50 ng), 12.5 μl 2× reaction buffer, a 0.2 μM concentration of both the forward and the reverse primer (Table 1), $MgSO_4$ to a final concentration of 5 mM, 0.5 μl Platinum *Taq* mix/SuperScript III and MilliQ. The RNA was incubated at 70°C and kept on ice before being added to the PCR mixture. The PCR conditions were as described for the DNA control, preceded by 15 min at 50°C.

Construction of *kshA* and *kshB* (co)expression plasmids for *E. coli* Bl21(DE3) and *E. coli* C41(DE3). All *kshA* and *kshB* genes were amplified via PCR using gene-specific primers from total DNA isolated from *R. rhodochrous* DSM43269 (Table 1). Gene cloning in expression vector pET15b and construction of the *kshA-kshB* coexpression plasmids pA1rho3 (*kshA1*), pA2rho2 (*kshA2*), pA3rho2 (*kshA3*), and pA5rho2 (*kshA5*) were performed as described previously (24) (see Table S1 in the supplemental material). The plasmid used for heterologous coexpression of *kshA4* and *kshB* has been described elsewhere (24).

Heterologous expression of KSH enzymes in *E. coli*, protein purification, and KSH enzyme activity assay. Coexpression and copurification of KshA and KshB (together constituting the KSH enzyme), as well as the KSH enzyme activity assay were essentially performed as described by Petrusma et al. (24). KSH activity was assayed by measuring the steroid-dependent NADH consumption over time. The assay mixture (total volume, 500 μl) consisted of 50 mM Tris-HCl buffer (pH 7.0), 25 to 40 μg of KSH enzyme, NADH (105 μM), and 200 μM steroid. NADH consumption was recorded with the Soft-max PRO4 (Life Science edition) program. KSH enzyme stocks were prepared and stored for a maximum of 5 days at a concentration of 1 to 2 mg ml^{-1} in 20% glycerol. The ratios of coexpressed and copurified $KshA_{DSM43269}$ homologues and KshB were determined using Tricine–SDS-PAGE (28) to separate the KshA and KshB components, followed by densitometry analysis. Uncoupling of the oxygenase enzyme reaction, which would result in NADH oxidation but not product formation, was checked by HPLC-UV (24) or gas chromatography (GC) analysis to confirm product formation. The formation of 9OHAD as a product of the reactions catalyzed by the KshA homologues incubated with AD was analyzed by electrospray ionization–LC-mass spectrometry (MS). Authentic 9OHAD (Schering-Plough, Oss, Netherlands) was used as a standard. Confirmation of 9OHAD formation was also performed using Δ1-KSTD, leading to the formation of 3-HSA as previously described (24). Authentic 3-HSA (Schering-Plough, Oss, Netherlands) was used as a standard.

Steroid analysis. Steroids were analyzed by HPLC-UV_{254} on an Alltima C_{18} column (250 by 4.6 mm, 5 μm) at 35°C using methanol-water (80:20) with 1% formic acid as a mobile phase at a flow rate of 1 ml/min. Samples (0.5 ml) were mixed with 2 ml mobile phase and filtered (0.2 μm) prior to analysis. Samples (0.5 ml) for GC analysis were mixed with 10% H_2SO_4 (10 μl) and ethyl acetate (2 ml), and the upper organic layer was subjected to GC. GC was performed on a 5% phenyl–5% methoxypoly-siloxane Heliflex AT-5 MS column (30 m by 0.25 mm, inner diameter, 0.25 μm; Alltech, Deerfield, IL) with FID-40 detection at 300°C.

RESULTS

Molecular characterization of five *kshA* homologues from *Rhodococcus rhodochrous* DSM43269. The five *kshA* genes, described elsewhere for *R. rhodochrous* DSM43269 (35a), are similar in length (1,137 to 1,200 nucleotides), share 65 to 70% identity at the nucleotide level, and encode proteins with 55 to 62% identity at the amino acid level. Bioinformatics analysis of the KshA homologues showed that all have the typical Rieske Fe_2S_2 binding domain (C-X-H-$X_{16,17}$-C-X_2-H) and the non-heme Fe^{2+} motif (D-X_3-D-X_2-H-X_4-H) (31). The eight amino acid residues predicted to be involved in binding of the steroid substrate in KshA of *M. tuberculosis* H37Rv, i.e., Val176, Gln204, Tyr232, Met238, Asn240, Asn257, Phe301, and Trp308 (H37Rv residue numbering per reference 4), were found to be fully conserved in the $KshA_{DSM43269}$ homologues, with the exception of Val176, which is an Ile in KshA1, and Asn240, which is an Asp in all $KshA_{DSM43269}$ homologues except KshA3.

Analysis of the genetic organization of the *kshA* homologues in DSM43269 revealed that *kshA1*, *kshA2*, and *kshA3* are located near genes annotated as steroid catabolic genes (Fig. 1A). The *kshA* genes of *R. rhodochrous* DSM43269 and the proximal genes all have orthologues in *R. jostii* RHA1 situated in one of the four previously described steroid catabolic gene clusters, although their genetic organization varied (Table 2) (19, 33). Interestingly, a *kshB* homologue, *kshB2*, was identified downstream of *kshA1*. KshB2 shares 66% amino acid identity with the KshB described previously (24). Adjacent to *kshA1*, *kshA3*, and *kshA4*, small putative genes encoding homologous proteins were found. An alignment of these protein sequences revealed the presence of four conserved cysteines, suggesting these genes may encode ferredoxin-type proteins (Fig. 1B) (18).

Next, phylogenetic analysis was performed on a total of 71 proteins sharing high similarity (>40%) with the KshA homologues of *R. rhodochrous* DSM43269 (Fig. 2). The phylogenetic tree showed clustering of these proteins into several groups. KshA2, KshA4, and KshA5 of strain DSM43269 appeared to be phylogenetically closely related proteins, belonging to the same group (Fig. 2). The other two $KshA_{DSM43269}$ homologues each belong to different groups.

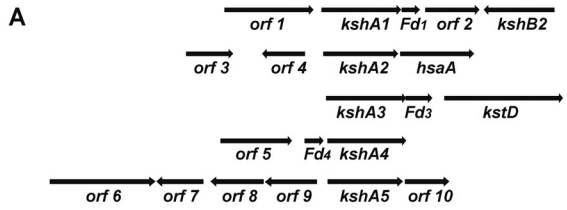

FIG. 1. (A) Schematic of the genomic organization of the *kshA* homologues of *R. rhodochrous* DSM43269. Annotation of the identified genes is provided in Table 2. (B) Alignment of the putative ferredoxin-type proteins (Fd) situated adjacent to *kshA1*, *kshA3*, and *kshA4*. *, conserved cysteine.

Physiological roles of *kshA* homologues in catabolism of different classes of steroids. To gain insight into the possible physiological roles of each of the $KshA_{DSM43269}$ homologues in steroid catabolism, growth experiments were performed with representatives of different classes of steroids, i.e., androstenes, sterols, pregnanes, and bile acids, by using AD, cholesterol, progesterone, or cholic acid, respectively, as a sole carbon and energy source. For this purpose, a 5-fold *kshA* null mutant of strain DSM43269, designated *R. rhodochrous* strain RG32, was constructed and it lacked *kshA1*, *kshA2*, *kshA3*, *kshA4*, and *kshA5* (35a). Contrary to wild-type DSM43269, the *kshA* null mutant strain RG32 showed no growth on the steroids tested, except for cholesterol (Fig. 3). Growth of strain RG32 on cholesterol was severely impaired, but not completely blocked. The slow growth of strain RG32 on cholesterol appeared to be supported by side chain degradation, as evident from the accumulation of ADD and 1,4-BNC, which is responsible for the release of propionate and acetate, which could be used as growth substrates (data not shown).

TABLE 2. Comparison of the genetic organization of *kshA* homologues of DSM43269 and neighboring genes with their orthologous genes in *R. jostii* RHA1

R. rhodochrous DSM43269 gene designation[a]	*R. jostii* RHA1 orthologue	RHA1 steroid catabolic gene cluster no.	Annotation in *R. jostii* RHA1	Amino acid identity (%)
*orf 1**	*ro05822*	3	Acyl-CoA synthetase	54
kshA1	*ro05811*	3	KshA3	72
Fd1	*ro09013*	4	Hypothetical protein	30
orf 2	*ro05810*	3	Dehydrogenase	74
kshB2	*ro05833*	3	KshB3	74
orf 3	*ro09032*	4	Short-chain dehydrogenase	82
orf 4	*ro09034*	4	IclR family transcriptional regulator	66
kshA2	*ro09003*	4	KshA4	72
hsaA	*ro09004*	4	HsaA4	79
kshA3	*ro04538*	1	KshA	75
Fd3	*ro04537*	1	Hypothetical protein	55
kstD	*ro04532*	1	KstD	76
*orf 5**	*ro04742*	1	Sensor kinase	61
Fd4	*ro02482*	2	Hypothetical protein	35
kshA4	*ro09003*	4	KshA4	57
orf 6	*ro02492*	2	Cyclohexanone monooxygenase	64
orf 7	*ro04889*		Short-chain dehydrogenase	42
orf 8	*ro02480*	2	Carveol dehydrogenase	64
orf 9	*ro02481*	2	Carveol dehydrogenase	66
kshA5	*ro02490*	2	KshA2	72
*orf 10**	*ro02498*	2	Aldehyde dehydrogenase	65

[a] Four steroid catabolic gene clusters have been identified in strain RHA1 (indicated by numbers 1 to 4). *, partially known gene sequence.

Next, the *kshA1*, *kshA2*, *kshA3*, and *kshA4* genes were individually reintroduced into strain RG32 under the control of their native promoter by using plasmid pRRE1 as a shuttle vector (26) (see Table S1 in the supplemental material). Mutant strain *R. rhodochrous* RG31, a 4-gene deletion mutant lacking *kshA1* to *kshA4*, was used to study the physiological role of *kshA5*. The growth experiments indicated that *kshA2*, *kshA3*, and *kshA5*, but not *kshA1* and *kshA4*, were able to restore the growth of mutant strain RG32 on cholesterol to a similar level as observed for wild-type strain DSM43269 (Fig. 3). The *kshA2* and *kshA5* genes also restored the growth of strain RG32 on both AD and progesterone. Again, *kshA1* did not restore growth of strain RG32 on these substrates, whereas the *kshA3* gene only partially complemented the growth on AD, progesterone, and cholic acid. Strain RG32 harboring *kshA4* was severely delayed in growth on progesterone, whereas full complementation was observed on AD. Interestingly, the growth of strain RG32 on cholic acid was restored only by *kshA1* and *kshA5*, strongly suggesting that *kshA1* is specifically involved in cholic acid catabolism. Growth on cholic acid was not restored by *kshA2*, *kshA3*, or *kshA4*.

In agreement with our observations in other studies (26, 35a), *kshA* null mutant strain RG32 was blocked in steroid ring degradation and accumulated the steroid pathway intermediates ADD and 1,4-BNC in cholesterol whole-cell bioconversions (Fig. 4). Subsequently, the role of each of the *kshA* homologues in cholesterol catabolism was studied in bioconversion experiments with cholesterol and the RG32 strains harboring the individual *kshA* genes (Fig. 4). The results showed that RG32 cell cultures expressing the *kshA2*, *kshA3*, or *kshA5* genes were able to degrade 1,4-BNC and ADD when

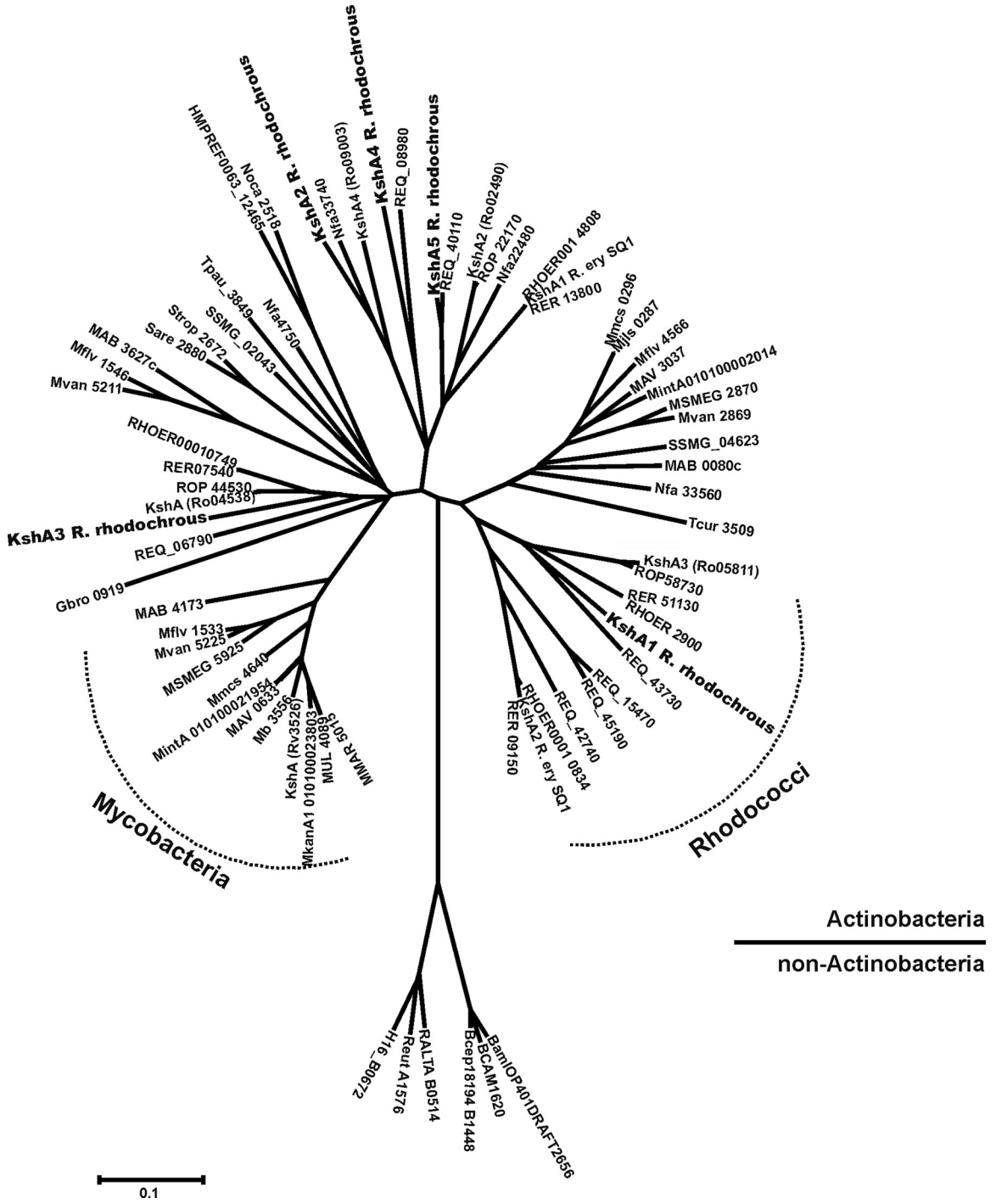

FIG. 2. Phylogenetic tree of bacterial $KshA_{DSM43269}$ enzymes and their orthologues in other actinobacteria and *Burkholderia* species.

incubated with cholesterol (Fig. 4). This is fully consistent with the results of the growth experiments using cholesterol as a substrate (Fig. 3). Strain RG32 harboring *kshA4*, however, still accumulated 1,4-BNC, similar to strain RG32 itself, but ADD was not detected. These results are in agreement with the inability of *kshA4* to complement growth on cholesterol as a sole carbon and energy source. In contrast, introduction of *kshA1* in strain RG32 enabled the partial degradation of 1,4-BNC, whereas ADD was not converted, consistent with the slowed growth observed on cholesterol by this recombinant strain.

None of the strain RG32 cell cultures with the various *kshA* genes accumulated novel pathway intermediates from cholesterol, which might have been indicative for a KshA enzyme with selectivity other than steroid 9α-hydroxylation.

Transcriptional analysis of the *kshA* homologues. To further substantiate the involvement of the 5 *kshA* homologues of

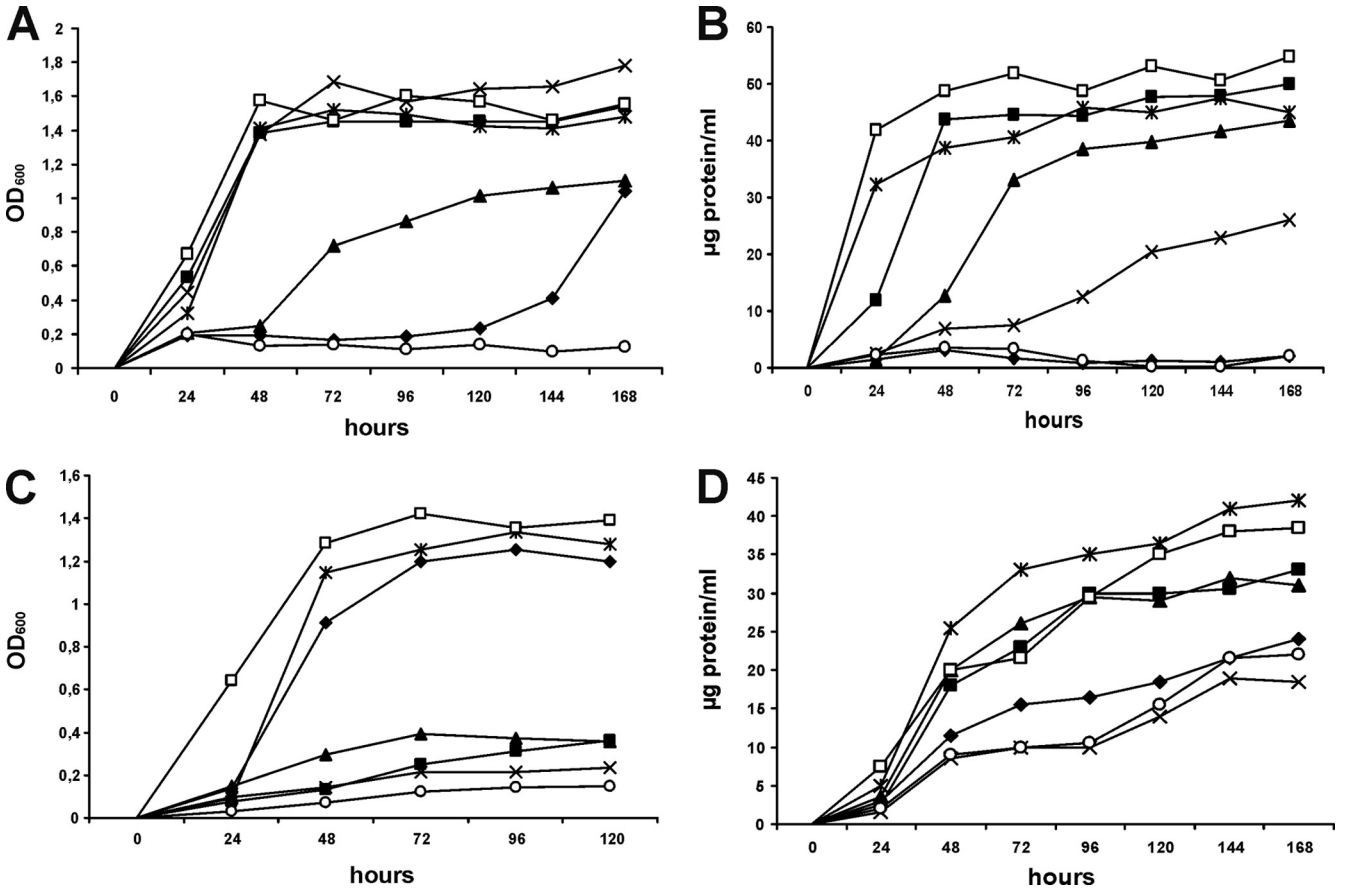

FIG. 3. Growth of *R. rhodochrous* DSM43269 wild-type strain (□), the *kshA* null mutant strain RG32 (○), recombinant strains RG32 transformed with *kshA1* (◆), *kshA2* (■), *kshA3* (▲), or *kshA4* (×), and the *kshA1 kshA2 kshA3 kshA4* deletion mutant strain RG31, still harboring *kshA5* (*) on 4-androstene-3,17-dione (A), progesterone (B), cholic acid (C), and cholesterol (D). Growth curves were performed in triplicate. See Table S2 of the supplemental material for standard errors of the means.

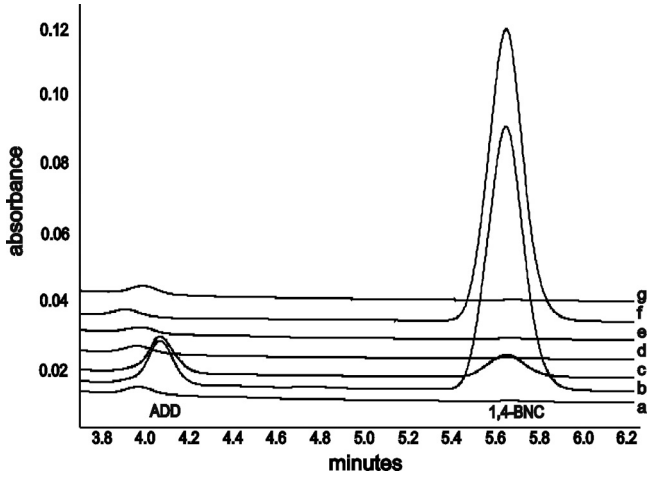

FIG. 4. HPLC elution profiles of cholesterol bioconversions by *R. rhodochrous* DSM43269 wild type (a), *kshA* null mutant strain RG32 (b), and recombinant RG32 strains transformed with either *kshA1* (c), *kshA2* (d), *kshA3* (e), *kshA4* (f), or mutant strain RG31 harboring *kshA5* (g).

DSM43269 in degradation of various classes of steroids, i.e., sterols, pregnanes, androstenes, and bile acids, we performed transcriptional analysis of cell cultures induced with cholesterol, progesterone, AD, or cholic acid, respectively. Wild-type *R. rhodochrous* strain DSM43269 cells were grown on acetate mineral medium and induced with cholesterol, progesterone, AD, or cholic acid. RT-PCR performed on isolated RNA from noninduced cultures showed that none of the *kshA* homologues was transcribed, indicating that none of the *kshA* homologues was constitutively expressed (Fig. 5). The transcriptional profiles of induced cells generally confirmed the results of our growth experiments: expression of *kshA1* is induced by cholic acid, and *kshA5* is induced by all steroids tested. Intriguingly, transcription of *kshA2* and *kshA4* was induced by cholic acid in wild-type *R. rhodochrous* DSM43269, although these KshA homologues did not appear to support growth on this bile acid when introduced in strain RG32. Transcriptional analysis of strain RG32 harboring either *kshA2* or *kshA4* induced with cholic acid indicated that both enzymes indeed are expressed in this *kshA* null mutant strain of *R. rhodochrous* DSM43269.

In wild-type *R. rhodochrous* DSM43269, *kshA3* was specifically induced by AD, whereas only partial complementation of growth of strain RG32 harboring *kshA3* on AD was observed.

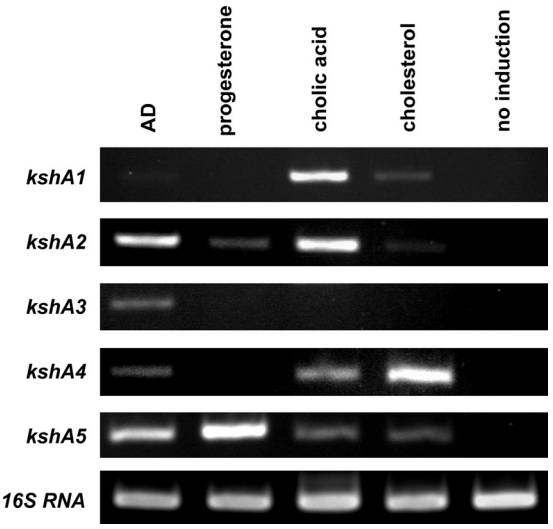

FIG. 5. Transcriptional analyses of wild-type *R. rhodochrous* DSM43269 cell cultures induced with AD, progesterone, cholic acid, or cholesterol. RT-PCRs were performed with gene-specific primers. 16S RNA served as a control.

Surprisingly, *kshA3* transcripts were not observed when wild-type DSM43269 cells were induced with cholesterol or progesterone, although *kshA3* is able to restore growth of strain RG32 on these steroids.

Heterologous expression and purification of KshA homologues of *R. rhodochrous* DSM43269. Based on the results of the growth experiments and the transcriptional profiling, differences in the substrate specificities of KSH enzymes with any of these 5 KshA homologues were expected. It also remained to be confirmed whether all 5 *kshA* homologues actually encode enzymes with 3-ketosteroid 9α-hydroxylase activity or steroid hydroxylases with a different substrate selectivity, e.g., as part of an alternative steroid catabolic pathway. The $KshA_{DSM43269}$ homologues therefore were individually expressed in *E. coli* together with the $KshB_{DSM43269}$ enzyme to determine their substrate preference and selectivity.

Cell extracts containing heterologously expressed KshA1, KshA3, or KshA5 all showed enzyme activities with KshB in a standard KSH activity assay using AD as steroid substrate. Consistent with our previous observations, the newly identified $KshA_{DSM43269}$ homologues could not be His tag purified in an active form (24). Using coexpression of KshA1 or KshA3 with KshB of strain DSM43269 in *E. coli*, and subsequent copurification, we succeeded in obtaining pure and active KSH enzymes. The molar ratios of KshA1:KshB and KshA3:KshB in the purified KSH fractions were 1:1 ± 0.16 and 1:0.72 ± 0.03 (means ± standard errors of the means), respectively. KshA5 could not be obtained in an active form when we used a single expression plasmid with both *kshA5* and *kshB*. When expressing KshA5 [*E. coli* BL21(DE3)] and KshB [*E. coli* C41 (DE3)] separately, followed by mixing of the two cell extracts, copurification resulted in an active KSH protein. The molar ratio of KshA5:KshB in the purified KSH fractions was 1:0.59 ± 0.04.

Unfortunately, no expression was observed with KshA2 in *E. coli* BL21(DE3) or *E. coli* C41(DE3) despite testing different expression (induction) times, growth temperatures, and isopropyl-β-D-thiogalactopyranoside concentrations for induction. Coexpression of KshA2 with KshB in *E. coli* BL21(DE3) or *E. coli* C41(DE3) also did not result in detectable amounts of KshA2 protein nor KSH activity when we used various steroid substrates.

Substrate preference and selectivity of KshA enzymes. The substrate preferences of the KSH enzymes comprised of KshA1, KshA3, or KshA5 copurified with KshB were determined in a KSH enzyme assay based on their initial enzyme activities on a range of different steroids (Table 3).

All $KshA_{DSM43269}$ homologues were able to use AD and ADD as substrates. However, substantial differences were observed in their substrate preferences. KshA1 and KshA3 showed higher activity on ADD than on AD, while AD was the preferred substrate for KshA5. Intriguing was the high preference of KshA1 for 4-BNC and 1,4-BNC. On the other hand, KshA5 had the broadest substrate range, with no apparent preference for any of the tested substrates, but it is unique in its ability to convert 5α-androstan-17β-ol-3-one (stanolon) and 11β-hydrocortisone. KSH activity was only observed with 3-ketosteroid substrates and not with 3-hydroxysteroid substrates (Table 3). The selectivity for C-9 α-hydroxylation of the AD substrate of all the $KshA_{DSM43269}$ homologues was confirmed by LC-MS analyses. Extracted steroid products from the reaction mixtures revealed the formation of 9OHAD from AD for all KSH enzymes. The extracted steroid products from each of the reaction mixtures were also incubated with purified KSTD1 enzyme of *R. erythropolis* SQ1 (13, 30). HPLC with diode array detection analyses confirmed the disappearance of 9OHAD and the appearance of 3-HSA (data not shown), the expected product of 9OHAD Δ1-dehydrogenation. These experimental data thus conclusively show that KshA1, KshA3, and KshA5 are 3-ketosteroid 9α-hydroxylase isoenzymes.

DISCUSSION

The well-known catabolic potential of rhodococci is greatly facilitated by the impressive gene multiplicity (15, 20, 32). Isoenzymes are involved in degradation pathways of several aromatic compounds, such as biphenyl, phenylacetate, benzoate, and phthalate in rhodococci (5, 8, 11, 22, 23, 27, 36). This study reports on multiple *kshA* genes, encoding the oxygenase component of KSH, found in *R. rhodochrous* DSM43269. A total of five *kshA* genes (*kshA1* to *kshA5*) have been identified (35a). They were shown to encode homologous $KshA_{DSM43269}$ enzymes with C-9 α-hydroxylation selectivity. Consistently, no activity was found with any of the $KshA_{DSM43269}$ homologues on 9OHAD. The latter would have been indicative of a KshA homologue with enzyme selectivity other than C-9 α-hydroxylation. This is further supported by the finding that novel hydroxylated steroid pathway intermediates from cholesterol were not detected in any strain of RG32 complemented with a *kshA* homologue of DSM43269.

Phylogenetic analysis of the five $KshA_{DSM43269}$ homologues revealed that these are distributed across the different phylogenetic groups that form the phylogenetic tree, although KshA2, KshA4, and KshA5 of strain DSM43269 appear to be phylogenetically closely related proteins (Fig. 2). The *kshA* genes of DSM43269 and their neighboring genes all have orthologues in *R. jostii* RHA1, each associated with one of the

TABLE 3. Steroid substrate ranges of four KshA homologues of *R. rhodochrous* DSM43269[a]

Steroid substrate	KshA1		KshA3		KshA4[c]		KshA5	
	Enzyme activity	Rel. activity (%)	Enzyme activity	Rel. activity (%)	Enzyme activity	Rel. activity (%)	Enzyme activity	Rel. activity (%)
4-Androstene-3,17-dione	$2.6 \times 10^2 \pm 10$	100	$1.7 \times 10^2 \pm 22$	100	$2.8 \times 10^2 \pm 20$	100	$1.5 \times 10^2 \pm 30$	100
1,4-Androstadiene-3,17-dione	$7.5 \times 10^2 \pm 90$	288	$3.3 \times 10^2 \pm 49$	194	$2.5 \times 10^2 \pm 20$	89	73 ± 11	49
4-Androstene-17β-ol-3-one	$3.3 \times 10^3 \pm 70$	127	$1.6 \times 10^2 \pm 40$	94	$2.8 \times 10^2 \pm 29$	101	$1.7 \times 10^2 \pm 24$	113
4-Pregnene-3,20-dione	$1.1 \times 10^3 \pm 94$	423	$1.9 \times 10^2 \pm 33$	112	$2.7 \times 10^2 \pm 38$	99	$1.0 \times 10^2 \pm 12$	67
19-Nor-4-androstene-3,17-dione	63 ± 7	24	55 ± 10	32	$2.2 \times 10^2 \pm 25$	78	$1.6 \times 10^2 \pm 25$	107
1-(5α)-Androstene-3,17-dione	59 ± 5	23	48 ± 5	28	$19 \times 10^2 \pm 20$	68	$14 \times 10^2 \pm 32$	93
5α-Androstane-3,17-dione	23 ± 13	9	ND	ND	$1.8 \times 10^2 \pm 19$	64	$12 \times 10^2 \pm 26$	80
5β-Androstane-3,17-dione	50 ± 7	19	ND	ND	$1.6 \times 10^2 \pm 22$	58	$1.4 \times 10^2 \pm 27$	93
5α-Androstane-17β-ol-3-one (stanolon)	ND	ND	ND	ND	ND	ND	$1.4 \times 10^2 \pm 27$	93
11β-Hydrocortisone	ND	ND	ND	ND	ND	ND	$1.4 \times 10^2 \pm 29$	93
4-Cholestene-3-one[b]	34 ± 5	13	39 ± 5	23	ND	ND	39 ± 5	26
23,24-Bis-nor cholesta-4-ene-22-oic acid	$1.3 \times 10^3 \pm 1.2 \times 10^2$	500	$2.1 \times 10^2 \pm 33$	124	—[d]	23	$1.2 \times 10^2 \pm 17$	80
23,24-Bis-nor-cholesta-1,4-diene-22-oic acid	$1.1 \times 10^3 \pm 1.3 \times 10^2$	423	$3.0 \times 10^2 \pm 44$	176	—[d]	22	64 ± 6	43

[a] Initial enzyme activities with a 200 μM steroid substrate concentration were calculated in nmol min^{-1} mg^{-1} of purified KSH enzyme. The relative activities are expressed as percentages compared to activities with 4-androstane-3,17-dione, which was set at 100%. Standard errors of the means ($n = 3$) are also reported. ND, no detectable initial activity. No KSH activity was observed with the following compounds: 5-cholestene-3β-ol (cholesterol), 5α-androstane-17-one, 3α-hydroxy-5α-pregnane-20-one, 3β-hydroxy-5α-androstane-17-one, 9α-hydroxy-4-androstene-3,17-dione (negative control).

[b] Due to its limited solubility, this steroid was used at 25 μM. (The lower concentration was also used for 5-cholestene-3β-ol and 3α-hydroxy-5α-pregnane-20-one [data are not shown].)

[c] Data were obtained from reference 24.

[d] Measurements were performed with enzyme batches with higher activities (initial activity on 4-androstane-3,17-dione of 512 ± 62 nmol min^{-1} mg^{-1} of purified KSH enzyme) than those described in reference 24 due to the slightly optimized expression and purification methods used in our study. However, the relative activities remained constant. For chemical structures of the tested steroids, see Fig. S1 in the supplemental material.

four steroid catabolic gene clusters (Table 2). Accordingly, we assigned $KshA3_{DSM43269}$ as a steroid cluster 1 type KshA, $KshA5_{DSM43269}$ as a cluster 2 type KshA, $KshA1_{DSM43269}$ as a cluster 3 type, and both $KshA2_{DSM43269}$ and $KshA4_{DSM43269}$ as cluster 4 type KshA enzymes. Two groups in the phylogenetic tree appeared to cluster at the genus level, with one group representing homologous proteins of *Rhodococcus* species and another group representing homologous proteins of *Mycobacterium* species (Fig. 2). Speculatively, these groups may represent specialized proteins providing catabolic potential to survive in a specific environmental niche. The *Rhodococcus* cluster, for example, may represent strains capable of growth on cholic acid, since it includes $KshA1_{DSM43269}$, a KshA specifically linked to cholic acid catabolism. The *Mycobacterium* cluster includes Rv3526 of *M. tuberculosis* H37Rv involved in cholesterol degradation (10, 33). Interestingly, KshA1 (Ro04538) of RHA1 is the orthologue of Rv3526 (33) but appears phylogenetically distinct from Rv3526. This may imply that cholesterol catabolism proceeds differently in *Mycobacterium* than in *Rhodococcus*, necessitating distinct KshA enzymes.

Bioinformatic analysis of the genetic organization in strain DSM43269 further revealed the presence of a small open reading frame (ORF), encoding a putative ferredoxin associated with three out of the five *kshA* genes of DSM43269 (Fig. 1B). Although KSH has been defined as a two-component enzyme by the classification system described by Batie et al. (2), these small ORFs may encode a third component of KSH. In the present study KSH activity was obtained when the KshA and KshB components were jointly expressed in *E. coli*, in the absence of these small proteins. However, a ferredoxin component could enhance efficient electron transfer between KshA and KshB. A small putative protein with four conserved cysteines is also found in close proximity to *kshA* (*ro04538*), *kshA2* (*ro02490*), and *kshA4* (*ro09003*) of *R. jostii* RHA1: *ro04537*, *ro02482*, and *ro09013*, respectively. Further studies are needed to verify a possible role for the putative ferredoxin proteins in KSH activity.

To provide insight into the functionality of the enzymatic multiplicity of the oxygenase component of KSH, we tried to unravel the physiological roles of each of the five KshA homologues in DSM43269. Enzyme activity assays showed that all KSH enzymes with $KshA_{DSM43269}$ homologues studied displayed KSH activity toward 3-ketosteroids (Table 3). KSH activity on 3-hydroxysteroid substrates was not observed with any of the KSH enzymes with $KshA_{DSM43269}$ homologues. KshA5 appeared the most versatile of the $KshA_{DSM43269}$ homologues. KshA5 had the broadest substrate range without a clear substrate preference and was active with Δ^4, $\Delta^{1,4}$, 5α-H, and 5β-H steroids, as well as with steroids having bulky aliphatic side chains. Moreover, expression of *kshA5* was induced by all steroids tested, and the 4-fold *kshA* mutant, carrying only *kshA5*, was capable of growth comparable to wild type on any of the steroid substrates tested (Fig. 3). KshA5 was also the sole KshA enzyme of strain DSM43269 capable of using 5α-androstane-17β-ol-3-one and 11β-hydrocortisone as substrates. Thus, it appears that the other four $KshA_{DSM43269}$ homologues are redundant for growth on steroids. The additional presence of the other KshA homologues, however, may aid in the efficiency of steroid degradation, allowing the bacterium to survive in a competitive environment, like soil. Indeed, it has been shown that the homologous enzymes of the

class IIA Rieske oxygenase carbazole 1,9α-dioxygenase of *Sphingomonas* sp. strain KA1 do not broaden the substrate range, but rather facilitate more efficient growth (29). It has also been suggested that the extensive gene redundancy in rhodococci makes the bacteria better equipped to adapt to new carbon sources (23).

The versatility of KshA5 is in strong contrast with KshA1, which appears to be dedicated to cholic acid degradation. The *kshA1* gene, but not *kshA2*, *kshA3*, or *kshA4*, complemented the growth of RG32 on cholic acid to the wild-type level (Fig. 3). In addition, *kshA1* was induced only during growth on cholic acid in DSM43269 (Fig. 5). KshA1 is a steroid cluster 3 type KshA, and we thus hypothesize that steroid cluster 3 in *R. jostii* RHA1 is involved in cholic acid. KshA1 is highly active on 4-BNC and 1,4-BNC compared to the other $KshA_{DSM43269}$ homologues (Table 3). Indeed, compounds such as 7α,12α-dihydroxy-23,24-bis-nor-cholesta-4-ene-22-oic acid and 23,24-bisnorcholesta-1,4-diene-22-oic acid have been found as intermediates in cholic acid degradation (3, 16). Expression of *kshA2* and *kshA4*, both encoding steroid cluster 4 type KshAs, was also induced by cholic acid in wild-type strain DSM43269 (Fig. 5). These genes, however, do not complement the RG32 phenotype in growth on this steroid (Fig. 3). Since the enzymes were expressed in the *R. rhodochrous* mutant strain RG32 background, it seems that the cholic acid catabolic pathway intermediates are not substrates for the KshA2 and KshA4 isoenzymes.

Gene expression of *kshA1* was also induced by cholesterol, albeit at a very low level. The *kshA1* gene appeared of no physiological relevance for growth on cholesterol: *kshA1* was unable to restore growth of strain RG32 on cholesterol, and accumulation of ADD and 1,4-BNC from cholesterol by a *kshA1*-complemented RG32 strain was still observed (Fig. 3), while both ADD and 1,4-BNC were preferred substrates of KshA1 (Table 3).

KshA3 is a steroid cluster 1 type *kshA* (Fig. 2). Steroid cluster 1 is named after the cholesterol catabolic gene cluster found in *R. jostii* RHA1 (33), thus suggesting that KshA3 is involved in cholesterol degradation. Indeed, *kshA3* restores growth of strain RG32 on cholesterol to the wild-type level, confirming a role for *kshA3* in cholesterol catabolism (Fig. 3). However, transcriptional analysis with the wild-type strain DSM43269 showed that *kshA3* is induced in the presence of AD, but not with cholesterol or any of the other steroids tested. We hypothesize that the formation of AD(D) in strain RG32 carrying *kshA3* induces *kshA3* expression, after which the substrate range of KshA3 allows full complementation of cholesterol degradation in strain RG32. This strongly implies that cholesterol degradation by wild-type DSM43269 is not accompanied by the formation of AD(D) able to induce *kshA3* expression. Several *kshA* homologues are induced by cholesterol in strain DSM43269 (Fig. 5), likely preventing the accumulation of AD(D) by premature ring opening and thereby *kshA3* expression during cholesterol catabolism. Intriguingly, *kshA4* is strongly induced by cholesterol but unable to restore growth on cholesterol (Fig. 5). The substrate profile for KshA4 indicated that this homologue has a subtle substrate range (24) (Table 3). Indeed, KshA4 is the only KshA with no detectable activity on cholestenone. In addition, both 4-BNC and 1,4-BNC are poor substrates (Table 3). Consistent with those findings, cholesterol bioconversions with a *kshA4*-complemented RG32 recombinant strain showed complementation in ADD degradation, but 1,4-BNC still accumulated (Fig. 4). The results taken together suggest that *kshA4* is induced by cholesterol but only acts in the degradation pathway after the side chain has been cleaved.

In conclusion, this study provides insight into the KshA multiplicity found in many *Rhodococcus* species. The KshA homologues of *R. rhodochrous* DSM43269 show high sequence similarity. They have the same enzymatic selectivity (C-9 α-hydroxylation) and also show overlap in their substrate range. However, the isoenzymes also show interesting differences, each displaying a unique induction pattern and substrate range. Furthermore, both the *in vitro* and *in vivo* studies indicated that the KshA homologues are involved in the degradation of specific steroids, acting at different levels in the steroid degradation pathway. The data suggest that the presence of multiple *kshA* genes in the *R. rhodochrous* DSM43269 genome facilitates a dynamic and fine-tuned response to the environmental changes encountered by the bacterium.

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